

Cloning, Expression, and One-Step Purification of the Minimal Essential Domain of the Light Chain of Botulinum Neurotoxin Type A

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A truncated but functional form of the botulinum neurotoxin A light chain (Tyr 9–Leu 415) has been cloned into the three bacterial expression vectors, pET 28, pET 30, and PGEX-2T, and produced as fusion proteins. This 406-amino-acid light chain was expressed with 1 six-histidine tag (LC-pET28), 2 six histidine tags and a S-tag (LC-pET30), or a six-histidine tag and a glutathione S-transferase tag (LC-pGEX-2T). The three fusion proteins have been overexpressed in *Escherichia coli*, purified in a soluble form, and tested for protease activity. All three recombinant proteins were found to have similar enzymatic activity, comparable to the light chain purified from the whole toxin. The LC-pET30 protein was the most soluble and stable of the three fusion proteins, and it could be purified using a one-step affinity chromatography protocol. The purified protein was determined to be 98% pure as assessed by SDS–polyacrylamide gel. This protein has been crystallized and initial X-ray data show that the crystals diffract to 1.8 Å. © 2000 Academic Press

Botulinum neurotoxins (BoNTs)² are produced by the rod-like, spore-forming bacterium *Clostridium botulinum*. Seven immunologically distinct forms of the neurotoxin, designated BoNT A through G, have been identified (1). BoNT, like its close relative tetanus toxin, affects nerve cells that control the action of muscles by preventing them from releasing synaptic vesicles filled with the neurotransmitter, acetylcholine, in-

ducing muscle paralysis. BoNTs enter these cells and inhibit acetylcholine release using a three-step process: binding of the toxins to cell surface receptors, internalization, and the poisoning or inhibition step that blocks neurotransmitter release (2).

The botulinum neurotoxins are synthesized as a single polypeptide chain (~150 kDa) which are activated by proteolytic cleavage to generate a dichain structure consisting of a heavy chain (~100 kDa) covalently linked through a single disulfide bond to a light chain (~50 kDa). The heavy chain is further divided into two domains, each with a specific function. The C-terminal half of the heavy chain (H_C) binds to the cell surface. The N-terminal domain (H_N) is thought to form “channels” in the endosomal membrane and to facilitate the transport of the light chain through the endosomal membrane into the cytoplasm. The light chain (LC) is the zinc-dependent catalytic domain of the toxin that targets and cleaves one of three SNARE proteins VAMP/synaptobrevin, Syntaxin, and SNAP-25 that are essential for synaptic vesicle fusion. Cleavage of the SNARE proteins results in inhibition of acetylcholine secretion, which leads to paralysis (3,4).

Although the sequences of the clostridial neurotoxins are highly homologous (50–60%), they all differ in their specificity for the SNARE proteins. BoNT/B, D, F, and G, as well as tetanus, cleave synaptobrevin (5), whereas BoNT/A and E act on SNAP-25 (6,7) and serotype C cleaves both SNAP-25 and syntaxin (8). In order to understand the factors responsible for the specificity these toxins exhibit toward the SNAP proteins as well as to be able to design effective inhibitors to the light chain, it is critical that we determine their structure and identify how they interact with their protein target. Recently, the crystal structure of the intact BoNT/A protein has been solved at a resolution

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² Abbreviations used: BoNT, botulinum neurotoxin; LC, light chain; GST, glutathione S-transferase.

of 3.3 Å (9). In the intact toxin, the light chain is buried in the protein with a loop from the translocation domain wrapped around it, partially blocking the active site in the unreduced form of the protein. It has been suggested that the location of this loop may explain why the catalytic activity of the intact toxin is greatly enhanced after treatment with reducing agents (4,10,11). Two different theories have been proposed to explain the increase in activity upon reduction. One theory suggests that the position of the large loop, or "belt," that wraps around the light chain is changed upon reduction, allowing access to the active site of the light chain. Another hypothesizes that the structure of the light chain, once it dissociates from the heavy chain, may be different. To obtain a better understanding of the catalytic activity of this protease, we have produced a recombinant form of the BoNT/A light chain for crystallization and structural analysis by X-ray diffraction. However, since the full-length light chain has been shown to have limited stability and solubility (personal communication, J. J. Schmidt and E. A. Johnson), we have constructed three expression vectors that produce a truncated form of the light chain with enhanced solubility and stability. This truncation is based on an earlier study in which the minimal essential domain of the light chain of BoNT/A was identified (12). In this study the minimal domain was determined by *in vitro* translation of various truncated mRNA deletion mutants. These experiments demonstrated that the deletion of 32 carboxyl-terminal and 8 amino-terminal residues from the light chain had no effect on its toxicity. Using this information, we produced truncated forms of the light chain by eliminating these 32 carboxy-terminal and 8 amino-terminal residues.

In this paper we describe the cloning of the truncated BoNT/A light chain into three *Escherichia coli* expression vectors and the expression of three recombinant forms of the protein containing a C-terminal 6×His-tag, an N- and a C-terminal 6×His in addition to a C-terminal S-tag, or a C-terminal 6XHis-tag and an N-terminal glutathione *S*-transferase (GST)-tag. The affect of the different tags on the protein's enzymatic activity, stability, and purification and conditions used for its crystallization are also reported.

MATERIALS AND METHODS

Cloning

The truncated LC region of the botulinum neurotoxin A cDNA, nucleotide positions 24–1245 corresponding to Tyr 9–Leu 415, was amplified by PCR and the amplified products were cloned into three expression plasmids to generate LC-pET28, LC-pET30, and LC-pGEX-2T expression constructs. Total genomic DNA from *C. botulinum* A (infant strain) (purchased

from E. Johnson) was used as the template in the PCR reaction to generate the LC-pET28 and LC-pET30 using the following primers: The 5' PCR amplification primer is 5'-AAACCCATGGCTTATAAAGATCCTGTAAATGGTGT-3' which adds a *Nco*I restriction site (indicated in bold) to the 5' end and the 3' PCR amplification primer is 5'-TGTAAGTCTGAGTAATCTAGTAAATTCCTGCTGT-3' which adds a *Xho*I restriction site (indicated in bold) to the 3' end. The 1245-bp product was cloned into the pET28b as well as pET30b (Novagen) expression vectors using the *Nco*I and *Xho*I restriction sites. The LC-pGEX-2T expression vector was generated by PCR amplification of the BoNT/A including the 6×His-tag at the carboxy end, using LC-pET28 construct as template. The 5' PCR amplification primer is 5'-TTAAGAAGAAGAGGATCCATGGCTTATAAAGATC-3' which adds a *Bam*HI site (indicated in bold) at the 5' end of the construct. The 3' PCR amplification primer is 5'-GTTAGCAGTGAATTCTCAGTGGTGGTGGT-3' which adds an *Eco*RI (indicated in bold) at the 3' end. The 1287-bp product was ligated into pGEX-2T vector containing the GST coding sequence (Pharmacia) using the *Bam*HI and *Eco*RI restriction sites.

The PCR reactions were performed in a total volume of 50 µl containing 60 mM Tris-SO₄ (pH 9.1), 18 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.2 mM each dNTP, 20 pmol of each primer, 100 ng of genomic DNA, and 1.0 unit of Elongase enzyme mix (Life Technologies). Reactions were heated at 80°C for 10 min before the DNA polymerase was added. After polymerase addition, reactions were cycled 31 times with 1 min denaturing at 94°C, 2 min annealing at 50°C (1 min in the last cycle), and 2 min extension at 68°C (6 min in the last cycle). The products were purified on quick spin columns (Qiagen, Chatsworth, CA) to remove excess primers. They were then digested with the appropriate restriction enzyme and were purified by agarose gel electrophoresis before ligation into the expression vectors.

Nucleotide Sequence Analysis

The nucleotide sequence of the three clones (LC-pET28, LC-pET30, and LC-pGEX-2T) were determined by Biotech Core (Palo Alto, CA) and found to be identical to the published sequences (13).

Expression and Purification of BoNT LC

Both LC-pET28 and LC-pET30 were expressed in *E. coli* BLR (DE3) cells (Novagen) and LC-pGEX-2T was expressed in *E. coli* JM109 cells (Promega). The transformed bacteria in each case were grown in LB medium at 37°C. Growth was monitored by absorbance measurements at 600 nm (OD₆₀₀) and at OD₆₀₀ = 0.6 expression was induced with 1.0 mM IPTG. The cells were grown overnight at 16°C. In a typical protocol

TABLE 1

Summary of Kinetic Values for SNAP-25 Hydrolysis by Native and Recombinant Light Chain

	K_m (mM)	V_{max} ($\mu\text{mol}/\text{min}/\text{mg}$)	k_{cat} (s^{-1})	k_{cat}/K_m
Native BoNT/A	5.0 \pm 0.5	1.9 \pm 0.5	4.7 \pm 0.2	0.94
Recombinant BoNT/A LC	0.76 \pm 0.07	0.104 \pm 0.011	0.087 \pm 0.01	0.096

cells were grown in 4 liters of LB medium and the harvested cells (wet weight ~ 4 g/liter) were either stored at -70°C or lysed on ice in 80 ml of Buffer A (25 mM Tris, pH 8.0, 100 mM NaCl, 1 mM 2-ME, 0.1% Triton X-100, 10% glycerol) containing 1 mM PMSF, EDTA-free protease inhibitor cocktail (Boehringer Mannheim), and 0.5 mg/ml lysozyme. After 1 h the cells were sonicated and then centrifuged at 20,000g for 30 min at 4°C . The supernatant was collected and was incubated with Ni-NTA agarose (Qiagen) resin (2.5 ml of resin for 1 liter of induced cells) equilibrated in Buffer A and stirred for 1 h to allow binding. The supernatant/agarose mixture was poured into a column and the bound protein and resin were washed with 10 column vol of Buffer A as well as 10 column vol of increasing concentrations of imidazole (10, 20, 40 mM) and 3 column vol of 90 and 250 mM imidazole. Fractions of the eluate were analyzed on 10% SDS-PAGE (Fig. 1).

The GST-LC protein expressed in the pGEX-2T expression vector was further purified by incubating fractions containing the LC from the Ni-NTA agarose column for 1 h with glutathione-Sepharose 4B resin (Pharmacia) while stirring. The resin was washed with 10 column vol of Buffer A and either cleaved with thrombin overnight at 25°C or eluted with 20 mM reduced glutathione before thrombin cleavage. The LC-pET28, which was also initially purified on Ni-NTA, was further purified on a Mono Q column (Bio-Rad) followed by size-exclusion column chromatography (Superdex-75). The LC expressed in pET30 eluted in the 90 mM imidazole fraction from the Ni-NTA agarose column and did not require further purification. This fraction was approximately 98% pure as judged by Coomassie-staining after SDS-PAGE.

Proteolytic Activity

The proteolytic activity of expressed light chain was assayed as described for whole type A neurotoxin (4), with a synthetic peptide substrate corresponding to residues 187–203 of SNAP-25. Before the assay, light chain was dialyzed against 25 mM Hepes buffer, pH 7.4. Kinetic constants were calculated from nonlinear regression analyses using the program Enzfitter (Bio-soft, Cambridge, UK), with data obtained from plots of initial rates of proteolysis vs seven different substrate

concentrations ranging from 0.3 to 2.3 mM (4,14). Results are averages of three independent determinations. Standard deviations were always less than $\pm 11\%$ (Table 1).

Crystallization

The LC-pET30 protein, with all tags intact, was used for the crystallization trials. The initial conditions were screened using the Crystal Screen I and II kits (Hampton Research) via the hanging drop vapor diffusion method. Crystals were first produced in 0.2 M ammonium sulfate, 0.1 M sodium acetate, pH 4.6, 25% PEG 4000 at 20°C . The crystals obtained were long, poorly formed rods with tapering ends. These conditions were optimized by the addition of 3% xylitol and storage at 4°C , producing long rectangular rods. Conditions from four different random screens, generated by the program CRYSTOOL (15), have been explored and new sets of conditions were identified. Current optimal conditions involve the use of 3 μl of protein solution (2–13 mg/ml in Buffer A), 2.4 μl of well solution (0.2 M ammonium sulfate, 0.1 M sodium acetate, pH 4.6, 25% w/v PEG 4000) and 0.6 μl 30% xylitol in deionized water. Crystals grew in about 3 weeks ($0.75 \times 0.2 \times 0.2$ mm) and diffracted to 1.8 Å. These conditions are being further refined.

Crystallographic Data Collection and Analysis

Synchrotron X-ray diffraction data were collected at the Advanced Light Source (Lawrence Berkeley Laboratory, Berkeley, CA) on beamline 5.0.2, with an ADSC Quantum 4 CCD detector. Crystals were washed briefly in a solution of mother liquor plus 25% glycerol, flash-cooled in liquid nitrogen, and mounted with cryotongs into a stream of 125°K nitrogen gas. Diffraction data were indexed and integrated with MOSFLM and scaled and merged with SCALA (16) of the CCP4 program suite. A molecular replacement solution has been found.

RESULTS AND DISCUSSION

Expression and Purification of the BoNT/A L Chain

The light chain of BoNT/A was cloned into three bacterial expression vectors. Our goal was to deter-

mine the best system for producing the most stable, soluble protein that could easily be purified in large quantities. These expression systems produced the light chain as a fusion protein containing either a 6×His-tag, two 6×His-tags in addition to an S-tag, or a 6×His-tag and a GST-tag.

Initially the LC was cloned into the expression vector pET28, which adds a 6×His-tag to the carboxy-terminal end of the LC. The protein was overexpressed in *E. coli* BLR (DE3) and the soluble fraction was bound to a Ni-NTA agarose column. The light chain was then eluted using an imidazole step gradient. The His-tagged fusion protein eluted at very low imidazole concentrations (20 mM) indicating that the binding to the column was very weak. The weak binding of the LC-pET28 protein to the column could be explained if the His-tag were inaccessible as a result of its being buried or tightly bound to the surface of the protein. To obtain the desired purity, the LC had to be further separated on a Mono Q anion-exchange column followed by a sizing column. This protein was not very stable and precipitated within a few days of storage at 4°C. Samples concentrated to 1–2 mg/ml precipitated almost immediately.

To increase the solubility and stability as well as to simplify the purification procedure, we added an additional GST-tag to the N-terminal end of the light chain. To generate this fusion protein, the light chain containing the C-terminal His-tag was amplified and cloned in frame with the GST gene in the pGEX-2T expression vector. This fusion protein was expressed in *E. coli* JM 109 and purified on a Ni-NTA affinity column followed by chromatography on a GSH-agarose column. A thrombin cleavage site is present between the LC and the GST, which allows the separation of the two components of the fusion protein. Although the addition of the GST tag improved the purification of the light chain, it did not have a significant affect on stability or

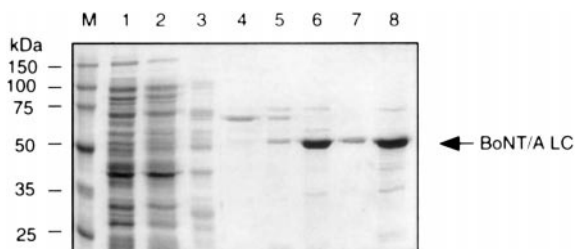


FIG. 1. Purification of the recombinant botulinum neurotoxin A light chain (LC-pET30) on a Ni-NTA affinity column. Proteins were separated on a 10% SDS-PAGE gel and Comassie blue stained. Lane M, perfect protein markers (Novagen); lane 1, flowthrough after nickel chelation of soluble fraction; lane 2, wash fraction with no imidazole; lane 3, 10 mM imidazole wash fraction; lane 4, 20 mM imidazole wash fraction; lane 5, 40 mM imidazole wash fraction; lane 6, 90 mM imidazole eluate; lane 7, second 90 mM imidazole eluate; lane 8, 250 mM imidazole eluate.

TABLE 2

Purification of the Botulinum Neurotoxin A Light Chain

Fraction	Total protein (mg)	LC (mg)	Purity of LC (%)	Yield (%)
Cleared lysate	90 ^a	2.5	2.7	
90 mM fraction	1.0	1.0	98	40

^a There were approximately 4 g wet wt cells used in a typical prep. The total protein listed is total protein in the clear lysate after sonication and centrifugation.

solubility of this protein. The stability of the LC with a GST and a His-tag was similar to the LC with only one His-tag; it also precipitated after storage for few days at 4°C. Attempts to remove the GST tag also resulted in precipitation.

Since the combination of a GST and a His-tag did not improve the stability of the LC, we cloned the sequence into another expression vector containing an N-terminal S-tag in addition to a His-tag on each end of the protein. We expected that the additional His-tag might not only help in the purification, but it might also improve stability of the light chain. To generate the two His-tag fusion protein, the LC was cloned into the pET 30 vector and it was expressed in *E. coli* BLR (DE3). This protein could be purified in a single step using the Ni-NTA column (Fig. 1). The column was washed at 10, 20, and 40 mM and eluted at 90 and 250 mM imidazole concentrations. The 90 mM imidazole fraction was determined to be enzymatically active and it could be purified to 98% homogeneity (Table 2). This fraction was very stable and after dialysis into Buffer A to remove the imidazole, the protein could be concentrated to a concentration as high as 6–12 mg/ml without precipitation. A substantial amount of the protein also eluted in the 250 mM imidazole fraction; however, this protein was not enzymatically active, presumably because it was not properly folded. The relative levels of overexpression with the three constructs tested were very similar. One critical difference, however was the stability of the purified proteins.

Proteolytic Activity and Stability of the Light Chain

The enzymatic activity of the recombinant LC, the LC prepared from the whole toxin, and native BoNT/A were compared by assaying the proteolytic cleavage of peptide [187–203] of SNAP-25. We have only presented the data for the recombinant LC since both the recombinant and the native LC prepared from the whole toxin had very similar activities based on the initial rates of hydrolysis with 1 mM substrate and 50 µg/ml enzyme. Kinetic constants for the light chain are summarized in Table 1, and corresponding values for the whole type A neurotoxin (4) are also included. The k_{cat}

TABLE 3

Effect of Mercaptoethanol, ZnCl₂, and DTT on the Proteolytic Activity of the Light Chain

	% Inhibition ^a
10 mM mercaptoethanol	56
20 mM mercaptoethanol	76
10 μ M ZnCl ₂	81
20 μ M ZnCl ₂	90
5 mM DTT	92
5 mM DTT + 20 μ M ZnCl ₂	70
5 mM DTT + 200 μ M ZnCl ₂	50

^a Inhibition of initial rate of proteolysis. Substrate was 1 mM and light chain was 50 μ g/ml.

for the recombinant light chain is quite low, about 2% of that for the native toxin. However, based on the catalytic efficiencies (k_{cat}/K_m), the LC has only 10-fold lower activity. The activity of the recombinant light chain based on initial rates of hydrolysis was similar to that of light chain prepared from intact toxin (data not shown). Taken together, these results suggest that some structural change in the free light chain may alter the catalytic efficiency of the active site. The presence of the loop or belt that has been shown by Lacy *et al.* (9) to wrap around the light chain and across the active site in the intact toxin would be expected to limit the entrance of the substrate into the active site, but its absence should have the opposite effect. Upon release of the light chain from the HC and removal of the belt, we would expect the k_{cat} to increase. The opposite affect was observed. Why the presence of the HC should increase proteolytic efficiency of the LC is not obvious, but the catalytic data we have obtained indicate that the LC must undergo some structural change upon its release that affects the turnover number of the protein. We have recently crystallized the recombinant LC and our data indicate that the LC is crystallized as a dimer. This observation may explain the lower activity of the LC in the absence of the HC.

Native botulinum neurotoxins require pretreatment with reducing agents for maximum expression of *in vitro* proteolytic activities (4,10,11,14). It is hypothesized that because the catalytic site of the light chain is occluded by the heavy chain, reduction of the inter-chain disulfide bond allows dissociation of the two chains, with a concomitant increase in activity. In consonance with this theory, we found that addition of 2-mercaptoethanol or dithiothreitol (0.02 to 5 mM) to assays of recombinant light chain, with or without added Zn²⁺ (0.01 to 0.10 mM), did not stimulate activity. In fact, such additions were inhibitory (Table 3).

We have also studied the stability of the LC upon storage and found that the recombinant LC is stable over prolonged periods at 4°C (Table 4). The activity of

the LC was monitored for a period of 43 days at 4°C in two different conditions. Sample A was stored in the purification buffer (Buffer A). It was not dialyzed, but was simply diluted 1:5 into 25 mM Hepes, pH 7.4 (Buffer B), just before assay. Sample B was dialyzed into Buffer B at the beginning of the experimental period. The activity of the LC in Buffer B was 17% higher than in Buffer A and the protein was found to remain active throughout the 43 days of storage. However, at the end of the 43 days the sample stored in Buffer A retained only 37% of its original activity. Buffer A contains Tris buffer and chloride ions, both of which are known to inhibit the proteolytic activities of the botulinum neurotoxins (4,11). The recombinant LC in Buffer B was also found to be stable to the freeze/thawing process.

Crystallization and Preliminary Crystallographic Analysis

Crystallization was observed in only 2 of about 200 experiments. The best reproducible crystals grew in a PEG 4000 solution at pH 4.6, in the presence of 3% xylitol. Diffraction data were collected. The space group is P2₁ (No. 4, $z = 2$) with unit cell dimensions of $a = 58.17$, $b = 94.53$, $c = 100.17$ Å, and $\beta = 103.55^\circ$. The Matthews coefficient (V_M) for a single molecule in the asymmetric unit is 5.34 Å³/Da; two molecules in the a.u. give a more likely V_M of 2.67 Å³/Da and a solvent content of 53%. The results of the initial crystallographic analysis are summarized in Table 5.

Examination of the native Patterson map (XtalView (17)) revealed a peak at the $y = 0.5$ Harker section indicating the presence of a noncrystallographic two-fold axis, positioned at $u = 0.125$ and $w = 0.224$. Further, preliminary molecular replacement calculations using coordinates from the intact toxin (provided by Dr. R. Stevens), using the program *epmr* (18), resulted in two nonoverlapping solutions. Both results support the presence of two molecules in the asymmetric unit.

TABLE 4

Net Rate of Proteolysis of SNAP-25 by Recombinant LC of BoNT/A

Days	Net rate (nmol/min/mg)	
	Buffer A	Buffer B
0	18.7 \pm 0.3	22.7 \pm 0.4
7	13.6 \pm 0.2	25.9 \pm 0.5
14	14.8 \pm 0.5	28.1 \pm 0.5
29	11.1 \pm 0.3	24.3 \pm 0.2
43	6.9 \pm 0.2	20.1 \pm 0.1

Note. Buffer A, 25 mM Tris, pH 8.0, 10% glycerol, 1 mM 2-ME, 0.1 M NaCl, 0.1% Triton X-100. Buffer B, 25 mM Hepes, pH 7.4.

TABLE 5
Data Collection Statistics for Light Chain from LC-pET30

Space group	P2 ₁
<i>a</i> (Å)	58.17
<i>b</i> (Å)	94.53
<i>c</i> (Å)	100.17
β (°)	103.55
<i>d</i> _{min} (Å)	2.00
Observed reflections	182181
Unique reflections	68242
<i>R</i> _{sym} (%) ^a	8.7
Data completeness (%)	96.1
⟨ <i>I</i> /σ⟩	5.7
Matthews coefficient <i>V</i> _m (Å ³ /Da)	2.67
Solvent content (%)	53.3
Molecules per asymmetric unit	2

^a $R_{\text{sym}}(I) = (\sum_{hkl} \sum_i |I_i(hkl) - \overline{I(hkl)}|) / (\sum_{hkl} \sum_i I_i(hkl))$, where *I_i* is the scaled intensity of the *i*th occurrence of the diffraction spot with coordinates (*hkl*).

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